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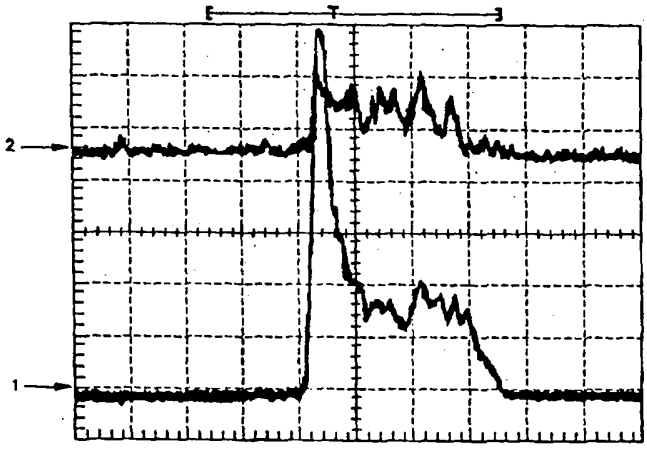
(54) Title: AXIAL PATTERN ANALYSIS AND SORTING INSTRUMENT FOR MULTICELLULAR ORGANISMS EMPLOYING IMPROVED LIGHT SCATTER TRIGGER



(57) Abstract

An improved instrument that consists of an optical analyser and a fluid switch using light scatter and fluorescence means to optically identify and activate fluidic sorting of multicellular organisms from live populations of organisms such as various life cycle stages of *Caenorhabditis elegans*, the larval stages of *Drosophila melanogaster*, and the embryonic stages of *Danio rero*. In the case where fluorescence from these organisms is very weak, comparatively high levels of electronic noise accompany the electronic signals that are generated by the fluorescence detector and its associated circuitry. Because these weak signals cannot be used to mark the presence of an organism, another, less noisy, signal must be used to gate fluorescence detection. A gate derived from the low-noise light scatter signal from the organism collected over an acceptance angle of at least 20 degrees. Such a light scatter signal unambiguously gates even weak fluorescence signals. These signals can then be correlated with position along the major axis of elongate, multicellular organisms and used as enhanced analysis and sorting parameters.

AXIAL PATTERN ANALYSIS AND SORTING INSTRUMENT FOR MULTICELLULAR ORGANISMS EMPLOYING IMPROVED LIGHT SCATTER TRIGGER

BACKGROUND OF THE INVENTION

The present application is based on and claims priority from U.S. Provisional Application No. 60/112,280, filed December 15,1998 which is incorporated herein by reference.

1. Field of the Invention

The present application concerns instruments to analyze and separate objects suspended in a fluid—specifically such instruments optimized to analyze and separate elongated multicellular organisms.

2. Description of Related Art

The present invention pertains to high-speed mechanisms for automatically identifying and physically selecting multicellular organisms with certain spatially distinct, optically detectable, phenotypic characteristics from mixed populations. Examples of applicable multicellular organisms are all stages of *Caenorhabditis elegans*, *Drosophila melanogaster* (fruit fly) larvae, or *Danio rero* (zebrafish) embryos. These are useful as model organisms for human disease and functional

The exposure of model organism mutants to diverse drug compound libraries, even when the specific mutations involved have not yet been linked to human gene homologues also helps define gene function. The addition of such functional genomic techniques to the repertoire of molecular biology and biochemistry methods can greatly accelerate the drug discovery process. Investigators can annotate drug libraries for toxicity, non-specific activity, or cell membrane permeability by observing their behavior in intact organisms. This way, toxic or ineffective libraries and/or library members can be discarded at an early stage without wasting valuable resources.

While model organisms such as the nematode *C. elegans*, the fruit fly *D. melanogaster*, and the zebrafish *D. rero* have been proven useful in the study of human disease, they have not yet been successfully used in the field of high speed, high throughput drug discovery. Until now high-speed preparation and analysis techniques have been missing for these large organisms. This presents a roadblock to investigators that need to search through thousands of multicellular organisms for a new mutation or for response to a given sample drug. For example, with today's molecular biology techniques, a large laboratory can produce deletion mutations in a multicellular test organism at a rate of 20 to 30 per month. Then, in order to evaluate the effect of a chemical compound library (that frequently contains 100,000 discrete compounds) on a class of mutated organisms, one must first manipulate and deposit a precise number of organisms of the mutant strain and the same development stage into various containers such as wells of a microtiter plate array. Wild type or deviants from

characteristics such as light scatter and fluorescence to classify each organism in a flowing stream. A single value of fluorescence intensity at a given emission wavelength is detected and assigned to each organism. The present invention is an improvement that enables a flow analyzer and sorter to localize and report not only the intensity but also the position of fluorescence along the major (long) axis of the organism and use this new spatial information to sort the organisms.

If a mutant strain or transgenic organism is characterized by a stable, spatial pattern of fluorescence, staining or other optically detectable characteristics, then the effect of therapeutic compounds or toxic environments on these strains can potentially be determined by monitoring changes in these spatial patterns. Discrimination of one pattern from another is currently carried out manually with the fluorescent microscope. This is an extremely tedious task requiring a significant number of workers that are trained at very high academic levels. Automating the detection of spatial patterns of fluorescence will improve the objectivity and the speed of measurement.

Flow instruments have been used before to count the number of nematodes in a fluid volume. Such a device was described by Byerly et al (L. Byerly, R.C. Cassada, and R.L. Russell, "Machine for Rapidly Counting and Measuring the Size of Small Nematodes", *Rev. Sci. Instrum.* Vol. 46, No. 5, May 1975) where the flow cytometer employed sheath flow to orient the nematodes along the direction of flow so that their size could be measured and organism-by-organism counts could be made by an electrical impedance method. The device was similar to a commercial Coulter counter.

fluorescence can be detected and recorded as a function of position along the length of the oriented organism (*i.e.*, an axial pattern scan). The invention is an improvement in speed and statistical precision over current manual techniques for analyzing multicellular organisms one by one under the microscope. The information from the scan can be used to characterize gene expression and enable physical selection and deposition of phenotypes with desired characteristics, or it can be used to determine alterations in gene expression caused by toxic or therapeutic compounds.

In the case where fluorescence from these organisms is very weak, comparatively high levels of electronic noise accompany the electronic signals that are generated by the fluorescence detector and its associated circuitry. These weak signals cannot be used to mark the presence of an organism, and another, less noisy, signal must be used to gate fluorescence detection. Axial light loss might be used as such a gate. Another preferred gate can be derived from the low-noise light scatter signal from the organism. Conventional light scatter gating, such as is practiced in flow cytometry of single cells, creates ambiguous signals when used on multicellular organisms and thus leads to false gating of fluorescence. A light scatter detection means is herein described which unambiguously gates these fluorescence signals. These signals can then be correlated with position along the major axis of elongate, multicellular organisms and used as enhanced analysis and sorting parameters.

Traditional optical flow cytometers analyze and sort small particles and single cells in liquid suspension by detecting light scatter within (over) narrow cone or solid

scatter" (LAFS) detectors are frequently placed as close as 0.5 degrees to the optical axis and collect light only within a one degree cone. Wide-angle light scatter detectors are frequently placed at positions ranging from approximately 10 degrees to 90 degrees off axis and also collect light within small cone angles of less than five degrees. If the cone angle of collection is not kept as small as possible, then information about granularity and size can become merged. Under these conditions for example, large cells become indistinguishable from small cells and granular cells become indistinguishable from non-granular cells of the same size.

When narrow acceptance cone light scatter (NACLS) detectors are used to monitor the passage of multicellular organism such as *C. elegans*, three problems arise that do not occur with single cells such as blood cells. First, it is found that the light scatter signal does not necessarily rise above baseline (zero) at the beginning of the passage of the organism through the optical beam, but instead rises at an unpredictably later time. Second, it is found that the light scatter signal does not necessarily return to baseline (zero) at the end of the passage of the organism through the optical beam, but instead returns at an unpredictably early time. Third, it is also found that the light scatter signal frequently returns to baseline (zero) at one or more unpredictable times while the organism is in the beam.

Therefore, the most basic effort to size multicellular organisms based on their "time of flight" through the analysis light beam is thwarted by this unpredictable behavior of light scatter signals that are collected over narrow cone angles.

sorting. For example, with *C. elegans*, it is important in many cloning applications to separate males from hermaphrodites. This can be accomplished with a fluorescently labeled lectin (wheat germ agglutinin) that binds to the vulva of the hermaphrodite and the copulatory bursa of the male. These two structures are not easily distinguishable in brightness, but the vulva is located near the midpoint of the organism and the copulatory bursa is located in the tail. Thus, axial location of fluorescence becomes the parameter for differentially analyzing and sorting males and hermaphrodites. This is illustrated schematically in Figure 1 where two oscilloscope traces are shown for single organisms. One trace (Fig. 1B) has a fluorescent peak near the midpoint, and the other (Fig. 1C) has a fluorescent peak at the tail.

Since there is no fluorescent signal to mark the beginning of the organism in the oscilloscope traces of Figure 1, a means must be established to mark the beginning and end of the passage of the organism through the light beam. This is done by the use of the wide acceptance cone, light scatter (WACLS) signal. The start of this signal triggers a clock in the electronic processor that, in turn causes fluorescent data to be sampled at regular intervals in time while the wide acceptance cone, light scatter signal remains above a preset threshold level. Sampling stops when the WACLS signal drops below threshold, denoting the end of the organism.

The following is a parametric representation of a multicellular organism that can be employed through the use of a WACLS signal to gate the sampling of fluorescence along the organism's axis. Consider a WACLS detector that produces

The fluorescence feature with emission wavelength F2 is not as small (along the axial direction) and occurs at a different location than the F1 feature. The relative location of the feature is established by reference to the timing initiated by the WACLS detector signal S1. If the velocity of the organism is known and the "tail" marker is used, then the absolute location of this feature can be determined as well. The fluorescence feature with emission wavelength F3 shows up in two small locations indicated in the WACLS timing sequence as T3 and Tn-1.

Each scanned organism can be represented by a parametric matrix of this kind. While not containing as much information as a microscope image of the organism, the data acquisition times for such matrices are of the order of five microseconds to 250 microseconds, depending on the length of the organism. This high speed is achieved because simple, fast photomultipliers collect the scattered light and no image is formed. In cytometers images are usually stored by CCD cameras, which are inherently less sensitive than photomultipliers, and therefore require more time to collect enough photons to form an image. Imaging times for fluorescence analysis of organisms such as *C. elegans* are of the order of 50 milliseconds, which is from 200 to 10,000 times slower than the time required to collect and store the parametric data described above. The sampling time and the speed of the organism determine the spatial resolution of the parametric method. For example, when the organism typically travels at about 500 cm/sec through the analysis beam, then for a five microsecond sampling time the spatial resolution is approximately 25 μm.

Figure 6A shows actual oscilloscope traces from a WACLS forward light scatter detector (lower trace) and a fluorescence detector at right angles to the optical axis (upper trace); the *C. elegans* samples scanned showed several discreet points of fluorescence.

Figure 6B shows actual oscilloscope traces from a WACLS forward light scatter detector (lower trace) and a fluorescence detector at right angles to the optical axis (upper trace); the *C. elegans* specimens scanned showed a small additional fluorescence at one end..

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

The following description is provided to enable any person skilled in the art to make and use the invention and sets forth the best modes contemplated by the inventor of carrying out his invention. Various modifications, however, will remain readily apparent to those skilled in the art, since the general principles of the present invention have been defined herein specifically to provide optical gating devices and methods for use with an optical analyzer/sorter designed for elongated multicellular organisms.

The flow scanning experimental system

An instrument such as that shown schematically in Fig. 1 was constructed with an interchangeable pair of lasers (argon ion and helium-neon) as the light source.

The traces show the passage of the organism through the line focus laser beam. The lower trace 1 is the light scatter signal and the top trace 2 is the fluorescence signal. The x-axis is time. Figure 4A is typical of a class of light scatter trace observed with a NACLS detector. The detector was placed at a 45 degree forward light scatter angle directly below the laser beam axis (below the horizontal plane in Fig. 2) as it emerged from the flow cell. No scattered light from the flow cell structures themselves was incident on the detector. The NACLS signal appears to rise at the proper time. The onset of the NACLS trace and the weak autofluorescence trace from the anterior structures of the nematode coincide. The NACLS signal appears to return to baseline after the fluorescent head passes. Unfortunately, the trace returns to baseline approximately during the middle of the passage of the nematode as well. This would give the false impression that two organisms had passed rather than one. This NACLS signal demonstrates the need for a new, unambiguous trigger and timing signal.

Figure 4B illustrates another problem associated with improper placement of a light scatter detector for triggering. In this example, the same detector was placed in the horizontal plane of Fig. 2, but at an angle of 45 degrees to the forward direction. In this case, stray, scattered light from the capillary was incident on the detector. A baseline restoration circuit was used to zero out this light level. The NACLS trace shows a false return to baseline that is caused by the acceptance cone angle being too small, and in addition a place where the signal becomes negative. The negative going region is caused when stray light from the flow cell is blocked by the nematode to an

several times wider cone angle than in the previous examples. The photomultiplier with a 40X collection lens and a barrier filter for green fluorescence protein was used to detect fluorescence since the fluorescence signal was very weak.

Figure 6A shows a WACLS signal on the lower trace and the associated fluorescence signal on the upper trace. Note that the WACLS signal begins and ends at the proper time and does not return to baseline during the passage of the nematode. This was a consistent and systematic observation so long as the acceptance angle was sufficiently wide and light from the illuminating beam or the scatter detector did not collect stray light. The particular *C. elegans* used for this example expressed fluorescence along its entire length with 5 to 6 points along the axis where the expression was locally stronger. Some evidence for these local peaks can be seen in the fluorescence trace. The WACLS signal begins and ends at the proper time and does not return to baseline during the passage of the nematode through the laser beam. There were no exceptions to this observation when over 500 nematodes were analyzed. In the examples of useless trigger signals described above almost half of the signals returned to baseline improperly.

Fig. 6B also shows the traces for a *C. elegans* with very weak fluorescent protein expression. There is a low level of autofluorescence throughout the length of the organism and two local regions of weak expression near the tail. The WACLS signal begins and ends at the proper time and does not return to baseline during the passage of the nematode through the laser beam. The fluorescence signal is far too

CLAIMS

What Is Claimed Is:

1. An instrument for analyzing and selectively dispensing elongate multicellular organisms comprising:

a source containing multicellular organisms in a fluid suspension;

means for causing the fluid suspension to move in a direction of flow;

means for aligning the elongate multicellular organisms relative to the direction of flow;

a light source for producing an optical beam through which the elongate multicellular organisms pass after becoming aligned;

a first optical detector for detecting light over a solid angle of at least 20 degrees for receiving light scattered by the elongate multicellular organisms for detecting passage of said organisms through said optical beam; and

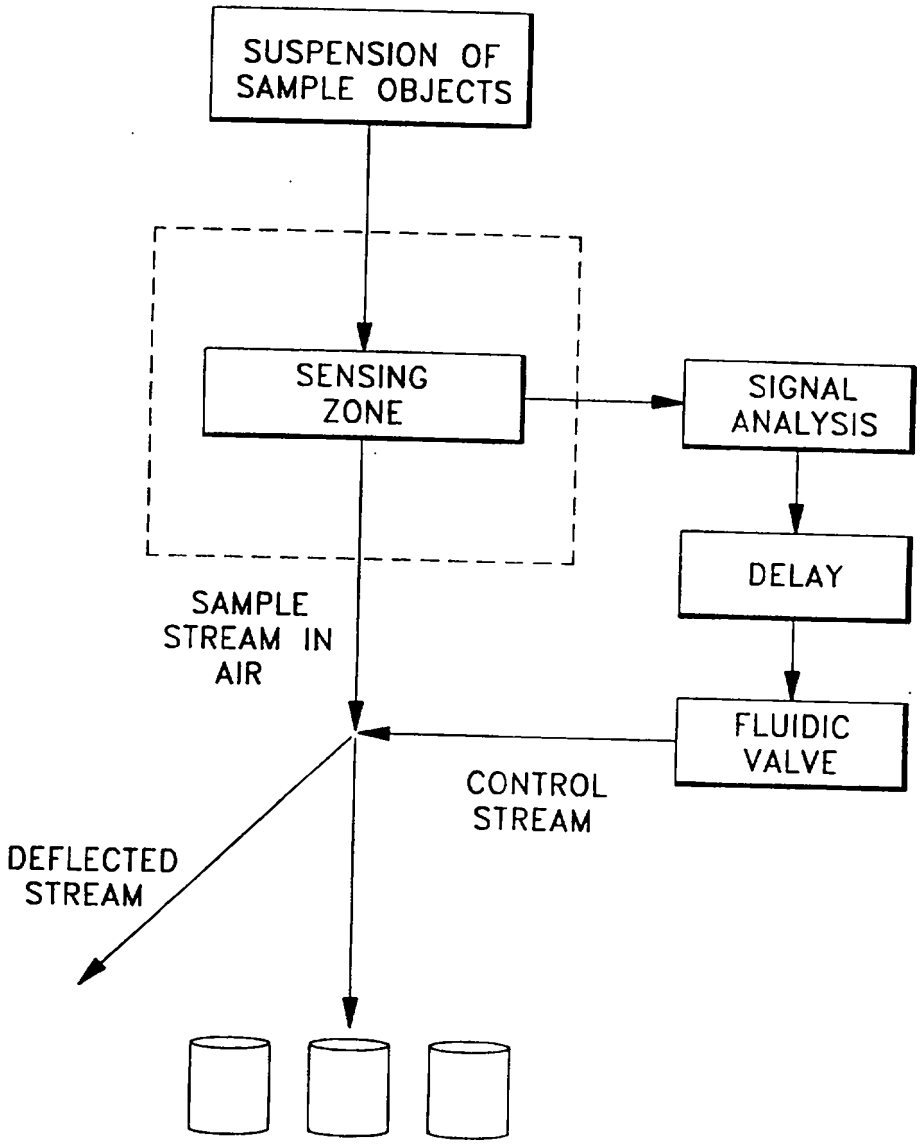
a fluid switch downstream of a point where said organisms pass through said optical beam, said switch responsive to the first optical detector to allow detected objects to pass to a sample container.

optically detecting the presence of a multicellular organism passing through the sensing zone by means of a light scatter sensor that has an acceptance angle of at least 20 degrees;

creating a data representation of sequential optical characteristics of the multicellular organism comprising output signals from additional optical sensors;

diverting at least some portion of the fluid stream with a switched fluid stream based on the data representation so as to collect ones of the multicellular organisms remaining in portions of the sample stream that were not diverted.

FIG. 1



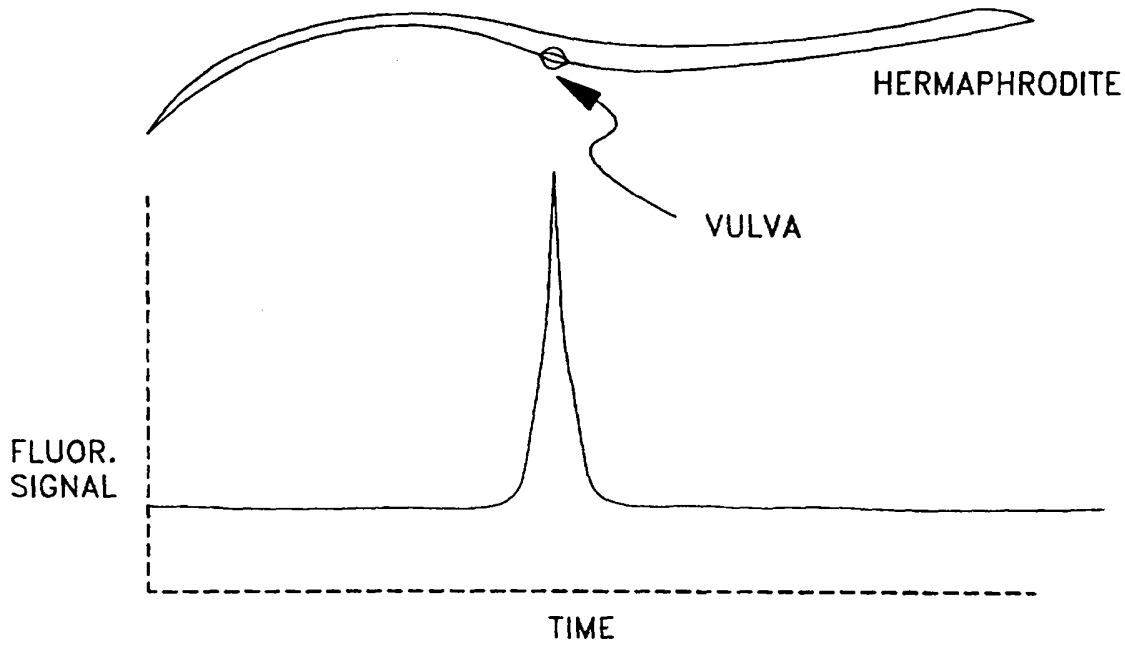


FIG. 3a

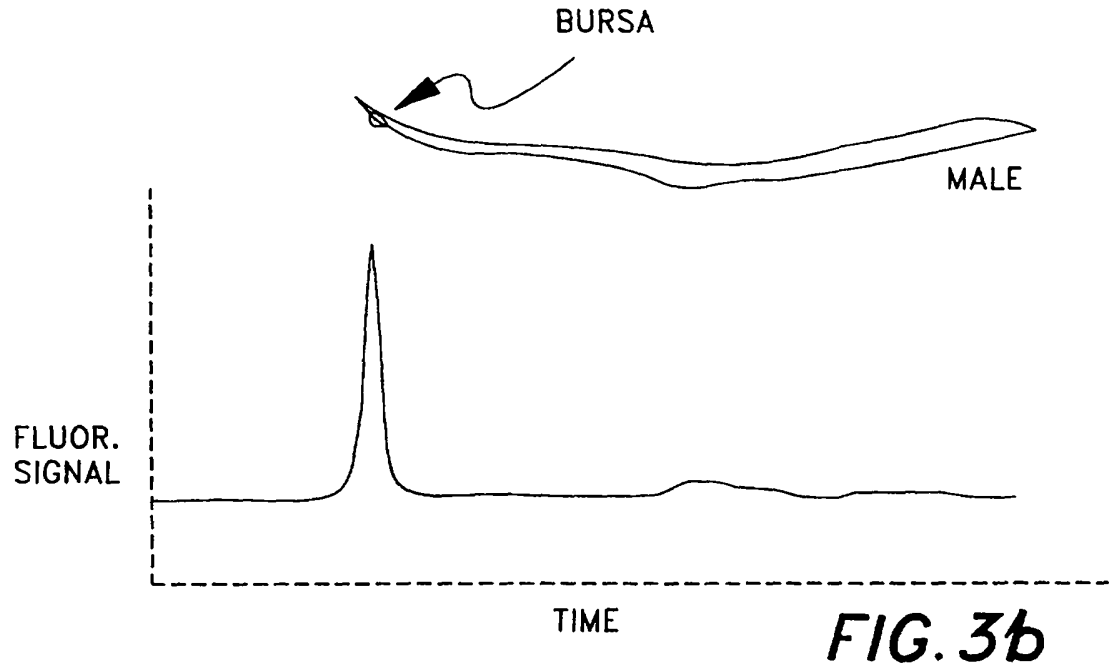


FIG. 3b

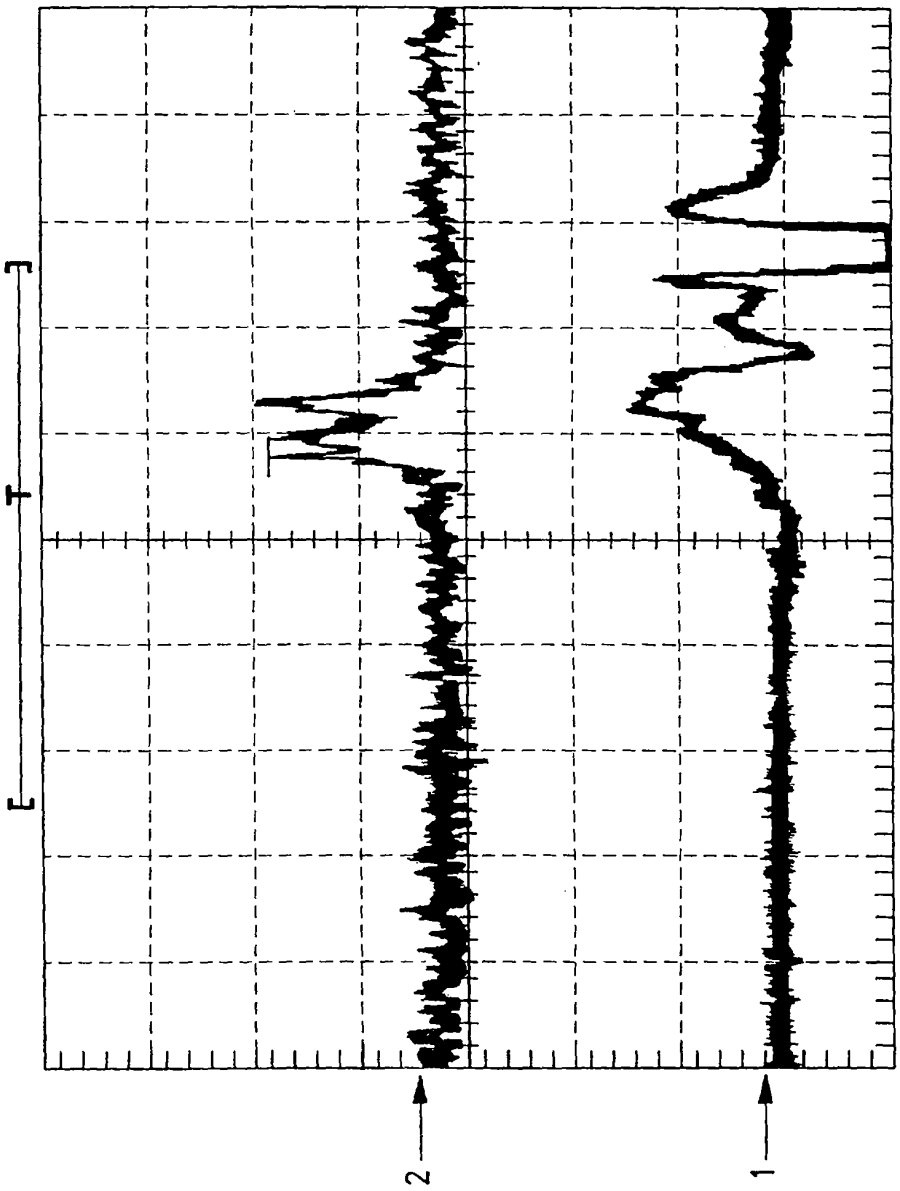
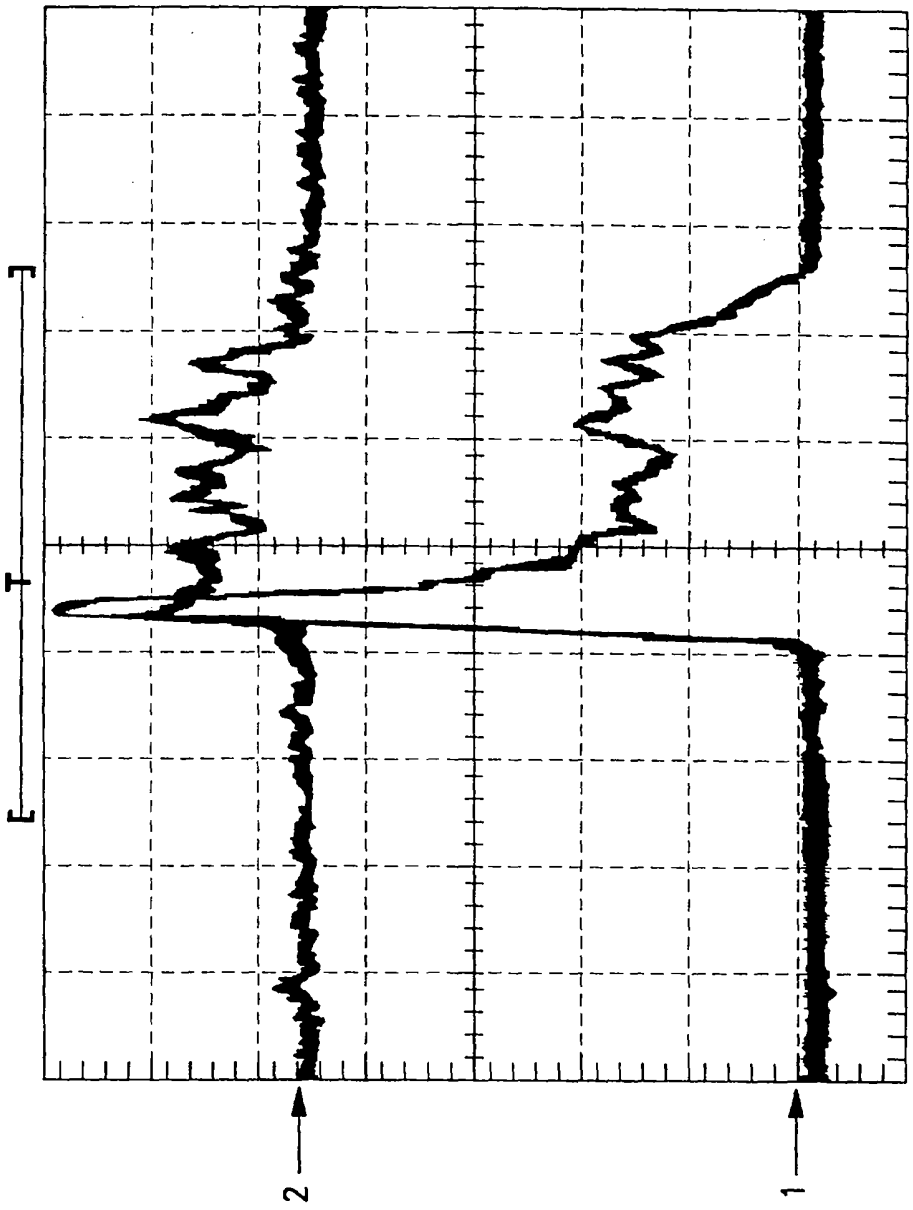


FIG. 4b

FIG.6a



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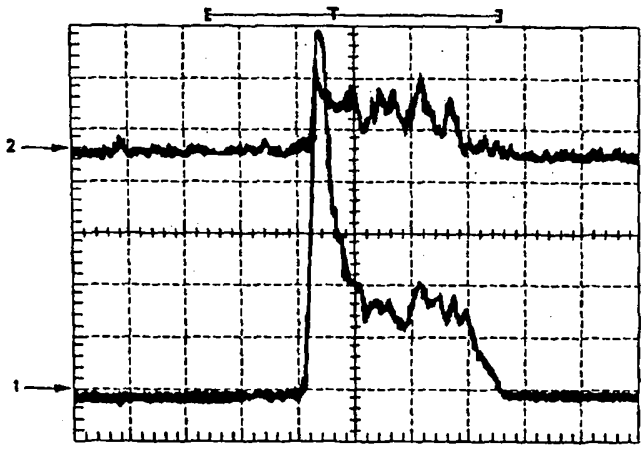

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INTERNATIONAL SEARCH REPORT

Interr nal Application No
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A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 G01N15/14

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 G01N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)
EPO-Internal, PAJ, INSPEC

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	US 5 798 222 A (GOIX PHILIPPE J) 25 August 1998 (1998-08-25) column 1, line 5-10 column 7, line 1-30 column 11, line 41,42 ---	1,5,8
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A	US 4 693 602 A (WYATT PHILIP J ET AL) 15 September 1987 (1987-09-15) column 2, line 20-37 column 4, line 64 -column 5, line 15 column 6, line 25-37 --- -/--	1,5,8

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

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Date of the actual completion of the international search	Date of mailing of the international search report
10 August 2000	18/08/2000

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL – 2280 HV Rijswijk Tel. (+31–70) 340–2040, Tx. 31 651 epo nl, Fax: (+31–70) 340–3016	Zinngrebe, U

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INTERNATIONAL SEARCH REPORT

Information on patent family members

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